SUPPLEMENTAL MATERIAL

APPENDIX A

Acquisition of Blood Samples Used for Assay of Analytes in the Present Study

The BARI 2D Fibrinolysis and Coagulation Systems Core Laboratory at the University of Vermont provided kits to all clinical sites on a regular basis throughout the duration of the study sufficient for sampling of all analytes. Site personnel were trained by the fibrinolysis and coagulation core personnel in careful venipuncture technique to ensure optimal compliance with OSHA standards, to minimize trauma to patients, and to optimize standardization of acquisition of samples to maximize accuracy of results. Detailed written instructions were provided that delineated procedures for collection of blood samples, separation of plasma, aliquoting, labeling of samples, and packaging and shipping of samples to the core laboratory. Plasma was separated by centrifugation within 15 minutes at 2000 g-min to ensure acquisition of plasma devoid of platelets which contain numerous constituents including PAI-1. Plasma was snap frozen in pre-labeled micro-centrifuge tubes and stored at -70°C until shipped by courier to the core laboratory in containers sufficient to hold 5 kg of dry ice. The kits provided by the core laboratory to the clinical sites contained vacutainer tubes, micro-centrifuge tubes, pipettes, storage containers for freezing and shipping samples, styrofoam shipping boxes, preprinted air bills to the core laboratory, cold packs, marking pens and IATA shipping labels. Once received on dry ice by overnight shipment in the core laboratory, each sample was assigned an accession number and placed in designated

and delineated storage spaces in a -80°C freezer until used for assays. Additional aliquots of each sample were stored in two separate freezers equipped with alarm systems to ensure adequate preservation of frozen samples. The alarm systems were connected to an automatic dialer that could alert personnel if temperature failures occurred outside of normal business hours.

All samples in the fibrinolysis and coagulation core laboratory were assayed by a personnel blinded to the patients from whom they had been obtained. Insulin, PAI-1 antigen, PAI-1 activity, and t-PA antigen were measured in each of the 13 samples obtained over 5 years from each patient (Table A). The sequence of sample acquisition was baseline, 1 month, 3 month, 6 months and every 6 months thereafter for a total of 5 years. Samples were drawn by peripheral venipuncture, placed on ice within 15 minutes, centrifuged for separation of plasma, frozen and shipped in batches to the Fibrinolysis and Coagulation Core Laboratory. At each time of sample acquisition, a sodium citrate tube was obtained for simultaneous acquisition of samples of separated plasma that would be banked. In a subset of patients who were studied in conjunction with a separate NIH grant supporting a BARI 2D ancillary study (Dr. Sobel, PI) samples were obtained over a 2-year interval at each clinical site and assayed in the fibrinolysis and coagulation core laboratory for FPA and D-Dimer, fibrinogen, and CRP (Table A).

The following numbers of samples were assayed in duplicate for each analyte respectively: 23,339; 23,273; 23,273; 23,339; and for insulin, PAI-1 antigen, PAI-1 activity, and t-PA antigen.. Samples from one clinical site for PAI-1 antigen and activity

were excluded because of artifact encountered in the sampling at that site pertinent to those two analyte sample tubes. For the analytes assayed in conjunction with the ancillary study the following numbers of samples were assayed in duplicate for FPA, D-Dimer, fibrinogen, and CRP: 2,328 at baseline, 2,006 at the end of one year, 1,341 at the end of the second year of follow up, respectively. The numbers of samples available in the ancillary study were less than those available for the study as a whole because samples after baseline were collected only during an 18 month period as pre-specified in the ancillary study NHLBI grant that supported acquisition and processing of these samples.. The numbers were lower after the baseline sampling interval because some patients were no longer available for sampling because of loss to follow up for sampling or mortality.

Processing of BARI samples: Persistent freezing of all samples was ensured performance of all sorting and labeling procedures while samples remained frozen over dry ice. A consecutive (ascension) number was assigned to each sample. The top of each tube was labeled with the last three digits of this number, each sample was sorted into one of a minimum of nine freezer boxes. The boxes were labeled with the range of all the numbers corresponding to all the samples contained within each box. Samples in the first 4 boxes were used for assays. Those in the second 4 boxes of citrated plasma samples were used for long term storage within 4 separate -80 degree freezers. In the ninth box, one sample of an unthawed heparinized plasma was banked.

Each assay of analytes was performed on samples that had not been previously thawed. The sample tubes were discarded after assays in duplicate were complete. The same procedures were applied to samples assayed for analytes in conjunction with the ancillary study.

Analytical procedures: Analytes were assayed as previously described in detail (1). The rationale for selection of analytes and the conceptual basis of the procedures used were as follows:

Assay of insulin: In the present study insulin was assayed as previously described (2). with the use of ALPCO Insulin ELISA kits providing a solid phase two-site enzyme immunoassay based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the human insulin molecule. During incubation insulin in the sample reacts with conjugated anti-insulin antibodies bound to microplate wells. The bound conjugates are detected by reaction with a TMB substrate. The reaction is stopped by adding acid, and the colorimetric endpoint is determined spectrophotometrically.

Assay of PAI-1 antigen: PAI-1 is the physiological inhibitor of plasminogen activator in blood (t-PA) and in tissues (urokinase type plasminogen activator). Increased concentrations of PAI-1 in blood are known to result in inhibition of fibrinolysis and a shift in the balance between fibrinolysis and thrombosis favoring thrombosis. For assay of PAI-1 in the present study, the Trinity Biotech TintElize PAI-1 procedure was utilized.

It is predicated on a double antibody principle, similar to that used in the ELISA developed initially by Declerck et al (3). It employs the same coating antibody as the Declerck assay (MA-7D4B7), quenching and non PAI-1 immune antibodies to exclude false elevations in results, an unfortunate confounder encountered with conventional ELISA assays.

Assay of PAI-1 activity: PAI-1 activity has the same physiological ramifications as the concentrations of PAI-1 antigen noted above. Assay of activity is entirely independent of assay of antigen. As noted in the results section, the close correlation of results obtained with these two separate procedures strongly supports the accuracy of both. For assay of PAI-1 activity in the present study, an excess of recombinant t-PA was added to human plasma at room temperature for use in reactions with samples. . The presence of PAI-1 in the samples, i.e., a high affinity inhibitor of t-PA, results in the binding of the t-PA. After incubations were complete the samples were acidified to terminate reactions and then snap frozen. Subsequently, remaining t-PA activity in thawed samples was quantified with the use of an indirect amidolytic method in which the generation of plasmin from plasminogen by plasminogen activator is reflected by the generation of para-nitroanilide with consequent absorbance at 405 nm resulting from cleavage of the plasmin-specific substrate, chromogenic substrate 2251 (4).

Assay of t-PA antigen: When concentrations of t-PA antigen exceed those of PAI-1 in blood such as is the case when t-PA or its congeners are administered pharmacologically to induce thrombolysis, the balance between fibrinolysis and

thrombosis is shifted markedly toward favoring fibrinolysis. However, under physiological conditions the molar content of PAI-1 in blood is generally two-fold greater than that of t-PA. Accordingly, no t- PA activity can be detected. For that reason and based on extensive preliminary analysis of samples from patients with and without diabetes, we elected a priori to measure t-PA antigen in the present study but not t-PA activity. It is well known that concentrations of t-PA in blood tend to parallel those of PAI-1 in part because of differences in clearance of free t-PA compared with clearance of t-PA complexed to PAI-1. In the present study we assayed t-PA antigen to determine whether a shift in concentrations increasing disproportionately to those of PAI-1 occurred in specific subsets of patients. Such a shift would be indicative of a shift in the balance of fibrinolysis and thrombosis favoring fibrinolysis. t-PA was assayed with the use of commercially available enzyme linked immunoassay kits (ELISA) from Trinity Biochech PLC (Bray Wicklow, Ireland) as previously described (1).

Assay of FPA: Fibrinopepeptide A (FPA) is a product of the action of thrombin on fibrinogen and a reflection of the initial reaction culminating in thrombosis. Accordingly, increased concentrations of FPA in blood are indicative of augmentation of thrombosis in vivo. However, in view of the very low values seen in plasma from normal subjects, the short half live of FPA in vivo, and the fact that FPA is markedly artifactually elevated in samples drawn under conditions in which venous occlusion is prolonged or tissue is mildly traumatized, its use as a reflection of the intensity of thrombosis in vivo is highly constrained. In the present study FPA was assayed with the use of the Vitro Chemie procedure, a competitive ELISA (5). Bentonite-treated plasma samples were incubated

with rabbit anti-FPA in microtiter wells coated with anti-rabbit IgG. FPA-biotin conjugates were then added to each well. The plates were washed and incubated with streptavidin-peroxidase conjugate. The concentration of FPA in each sample was then determined by incubation with the substrate TMB followed by the addition of hydrochloric acid. This procedure generates a yellow color. Because the procedure employed is a competition reaction, the concentration of FPA is inversely proportional to the absorbance read at 450 nm.

D-Dimer is a product of plasmin acting on a fibrin clot. Its concentration in blood increases in association with accelerated thrombosis such as that seen with disseminated intravascular coagulation (DIC) and deep vein thrombosis (6). D-Dimer was assayed in the present study to provide additional information regarding the relative intensity of thrombosis in patients in different subsets. The assay was performed with the use of the STAR automated coagulation analyzer, (Diagnostica Stago) and an immuno-turbidometric assay (Liatest D-DI; Diagnostica Stago, Parsippany, NJ). The reagents used in this assay are latex particles coated with two different mouse anti-human monoclonal antibodies specific to D-Dimer. Following addition of these reagents to plasma samples, antigen/antibody complexes are produced with the rate of change in absorbance over time reflecting the concentration of D-Dimer in the sample. Patient samples are validated with the use of two controls (6).

Assay of fibrinogen: Increased concentrations of fibrinogen are known to be associated with an increase proclivity toward thrombosis. Concentrations of fibrinogen were

assessed in the present study to identify a potential shift in the balance between fibrinolysis and thrombosis favoring thrombosis with the use of the STAR automated coagulation analyzer (Diagnostica Stago, Parsipanny, NJ), and the Clauss procedure (7). The clotting time of a diluted plasma sample in the presence of excess thrombin is a direct reflection of the concentration of fibrinogen in the sample under the assay conditions used.

Assay of C-reactive protein: As noted in the discussion section of the manuscript a considerable body of information implicates augmented intensity of the systemic inflammatory state as an etiological factor in acceleration of atherosclerosis and coronary events. To assess the relative intensity of the inflammatory state in subsets of patients in the present study, we assayed CRP in blood with the use of a BNII nephelometer from Dade Behring and a particle enhanced immunonepholometric assay (8). Polystyrene particles were coated with monoclonal antibodies to CRP, which, in the presence of antigen (CRP) agglutinate to cause an increase in the intensity of scattered light. The increase in scattered light is proportional to the amount of CRP in the sample.

Appendix A: References

1. McBane RD II, Hardison RM, Sobel BE (BARI 2D Study Group). Comparison of plasminogen activator inhibitor-1, tissue-type plasminogen activator antigen, fibrinogen, and D-Dimer levels in various age decades in patients with type-2 diabetes mellitus and stable coronary artery disease (From the Bypass Angioplasty Revascularization Investigation 2 Diabetes Trial). Am J Cardiol. 2010; 105:17-24.

2. Kruszynska YT, Yu JG, Olefsky JM, Sobel BE. Effects of Troglitazone on Blood Concentrations of Plasminogen Activator Inhibitor 1 in Patients With Type 2 Diabetes and in Lean and Obese Normal Subjects. Diabetes. 2000; 49:633-639.

3. Declerck PJ, Alessi M-C, Verstreken M, Kruithof EKO, Juhan-Vague I, Collen D. Measurement of plasminogen activator inhibitor 1 in biological fluids with a murine monoclonal antibody-based enzyme-linked immunosorbant assay. Blood. 1988; 71:220-225.

4. Chmielewska J, Wiman, B. Determination of tissue plasminogen activator and its “fast” inhibitor in plasma. Clin Chem. 1986; 32:482-485.

5. Soria J, Soria C, Ryckewaert JL. A solid phase immuno enzymological assay for the measurement of human fibrinopeptide A. Thromb Res. 1980; 20:425-435.

6. Levy G.: “Interet et limite du dosage d’un produit de degradation de la fibrine: le D-Dimere.” Sem Hôp Paris. 1987; 63/25:2061-2064.

7. Clauss A. “Gerrinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens”. Acta Haematol. 1957; 17:237-246.

8. Lakoski SG, Cushman M, Palmas W, Blumenthal R, D-Agostino RB Jr, Herrington DM. The relationship between blood pressure and C-reactive protein in the Multi-Ethnic Study of Atherosclerosis (MESA). J Am Coll Cardiol. 2005; 46:1869-1874.

Appendix A: Table A. Samples Collected and Assayable

Sampling interval	Core samples (PAI-1 antigen, & activity, t-PA, insulin)	Ancillary study samples (CRP, FPA, D-dimer, fibrinogen)
Baseline	2328	2303
Month 1	2081	
Month 3	2119	
Month 6	2080	
Year 1	2064	1984
Year 1.5	1920	
Year 2	1972	1350
Year 2.5	1798	
Year 3	1849	
Year 3.5	1502	
Year 4	1586	
Year 4.5	908	
Year 5	978	

Two types of samples were acquired: 1) core samples from all 2328 patients beginning at baseline, and 2) ancillary study samples from 2303 patients beginning at baseline. The percentage of samples that were acquired and suitable for analysis (i.e., not hemolyzed or clotted) of each type are shown for each time point of sampling. Declines in sample numbers over time for core samples reflected mortality, morbidity and missed BARI 2D clinic visits, loss to follow-up, and completion of the study before completion of

5-year follow-up for some patients. Declines for ancillary study sample numbers over time reflected all but the last of these factors. There were no appreciable differences in the percentages of samples from patients in the IS and IP treatment strategy arms over time (approximately 50% in each group at all intervals).

APPENDIX B

LEGENDS FOR FIGURES IN APPENDIX B

Figure 1: Means and median values of the analytes specified over time in the BARI 2D population as a whole. N = number of patients. IQR = interquartile range. SD = standard deviation.

Figure 2: Means and median values of the analytes specified over time in the BARI 2D population as a whole. N = number of patients. IQR = interquartile range. SD = standard deviation.

Figure 3: Kaplan-Meier 5 year rate estimates for mortality and Death/MI/Stroke by baseline values of the analytes specified.

Appendix B: Table A. Correlations between Year 1 analytes

Spearman correlation p-value n	t-PA	PAI-1 Antigen	PAI-1 Activity	CRP	FPA	D-Dimer	Fibrinogen	Insulin
t-PA								
PAI-1 Antigen	**0.5617** < 0.0001 1970							
PAI-1 Activity	**0.5081** < 0.0001 1978	**0.8337** < 0.0001 1971						
CRP	**0.2737** < 0.0001 1969	**0.3026** <0.0001 1879	**0.2648** <0.0001 1886					
FPA	**0.1006** <0.0001 1980	**0.1446** <0.0001 1889	**0.0676** 0.0032 1897	**0.1767** < 0.0001 1966				
D-Dimer	**-0.0055** 0.8085 1967	**-0.0657** 0.0044 1877	**-0.1478** < 0.0001 1884	**0.1966** < 0.0001 1962	**0.0989** <0.0001 1964			
Fibrinogen	**0.1489** <0.0001 1976	**0.05154** 0.0252 1885	**0.0184** 0.424 1892	**0.4468** <0.0001 1969	**0.1617** <0.0001 1971	**0.1974** <0.0001 1968		
Insulin	**0.3456** **<0.0001** **1941**	**0.3624** **<0.0001** **1857**	**0.3883** **<0.0001** **1861**	**0.2468** **<0.0001** **1853**	**0.1249** **<0.0001** **1868**	**-0.0002** **0.99** **1852**	**0.1403** **<0.0001** **1858**	

HbA1c	**0.1329** **<0.0001** **1974**	**0.1239** **<0.0001** **1883**	**0.1452** **<0.0001** **1890**	**0.1799** **<0.0001** **1890**	**0.0090** **0.70** **1900**	**-0.0095** **0.68** **1888**	**0.1314** **<0.0001** **1894**	**0.1130** **<0.0001** **1862**

Figure 1

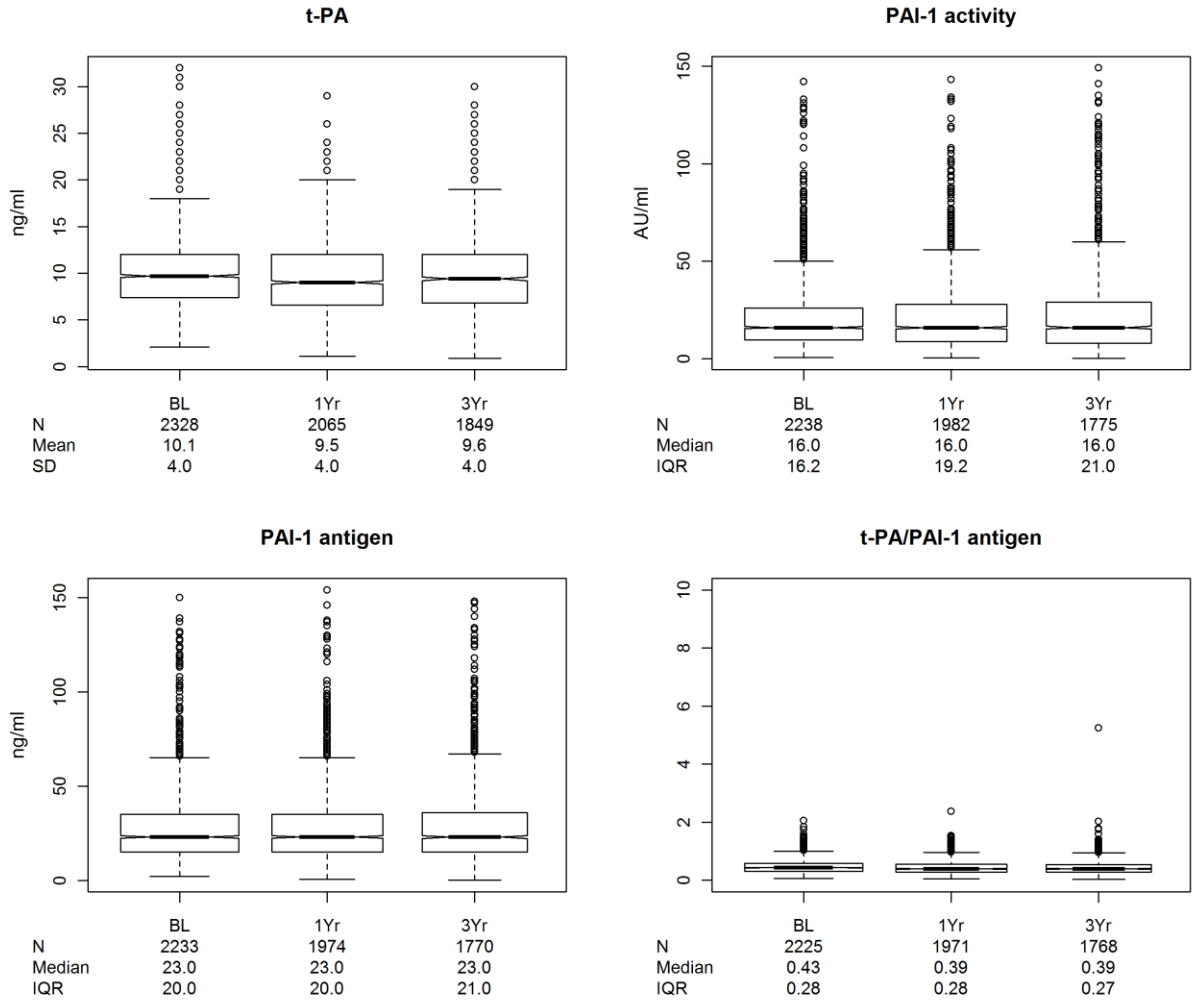

t-PA
ng/ml
0
5
10
15
20
25
30
BL
1Yr
3Yr
N 2328 2065 1849
Mean 10.1 9.5 9.6
SD 4.0 4.0 4.0
PAI-1 activity
AU/ml
0
50
100
150
BL
1Yr
3Yr
N 2238 1982 1775
Median 16.0 16.0 16.0
IQR 16.2 19.2 21.0
PAI-1 antigen
ng/ml
0
50
100
150
BL
1Yr
3Yr
N 2233 1974 1770
Median 23.0 23.0 23.0
IQR 20.0 20.0 21.0
t-PA/PAI-1 antigen
0
2
4
6
8
10
BL
1Yr
3Yr
N 2225 1971 1768
Median 0.43 0.39 0.39
IQR 0.28 0.28 0.27

Figure 2

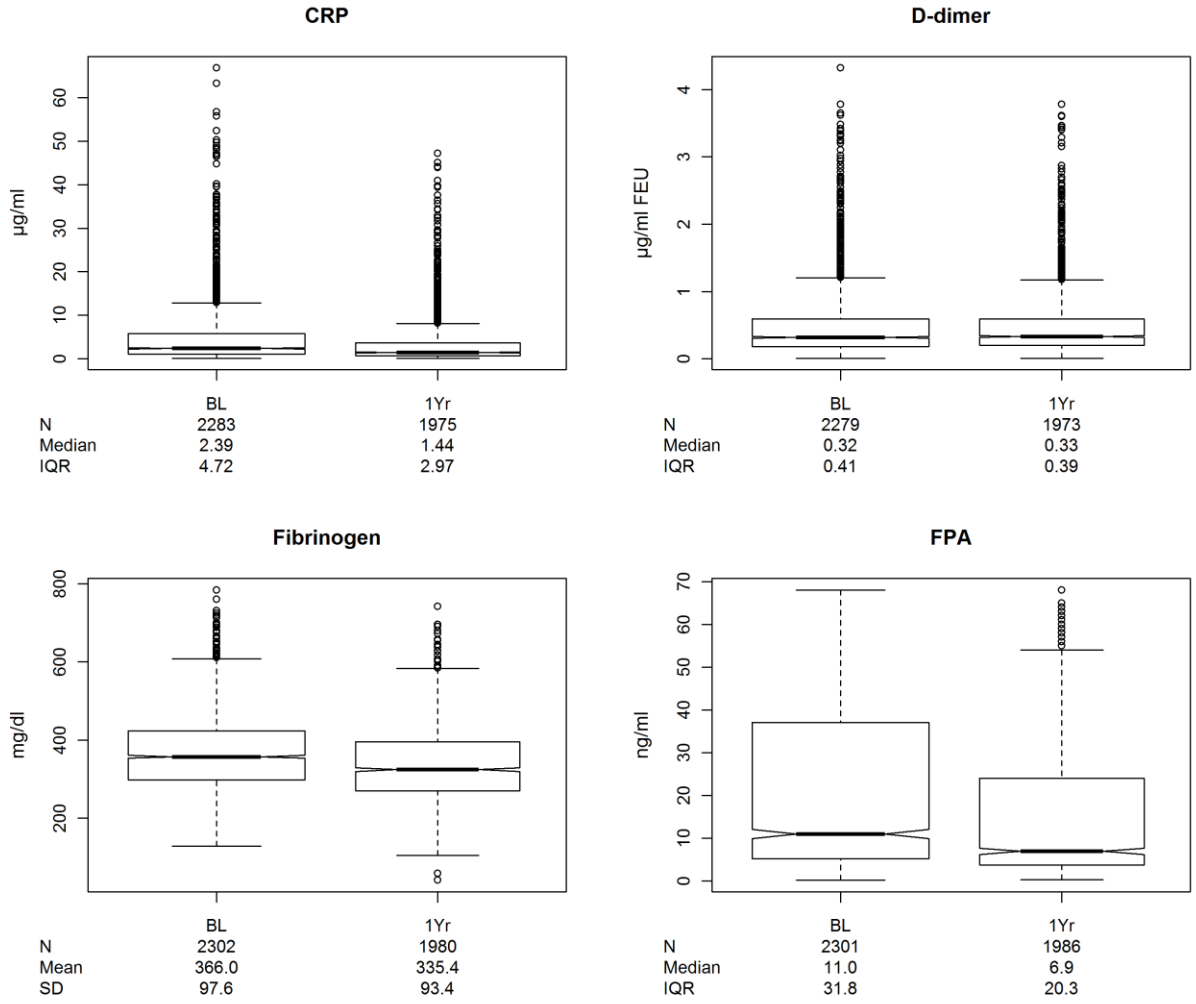
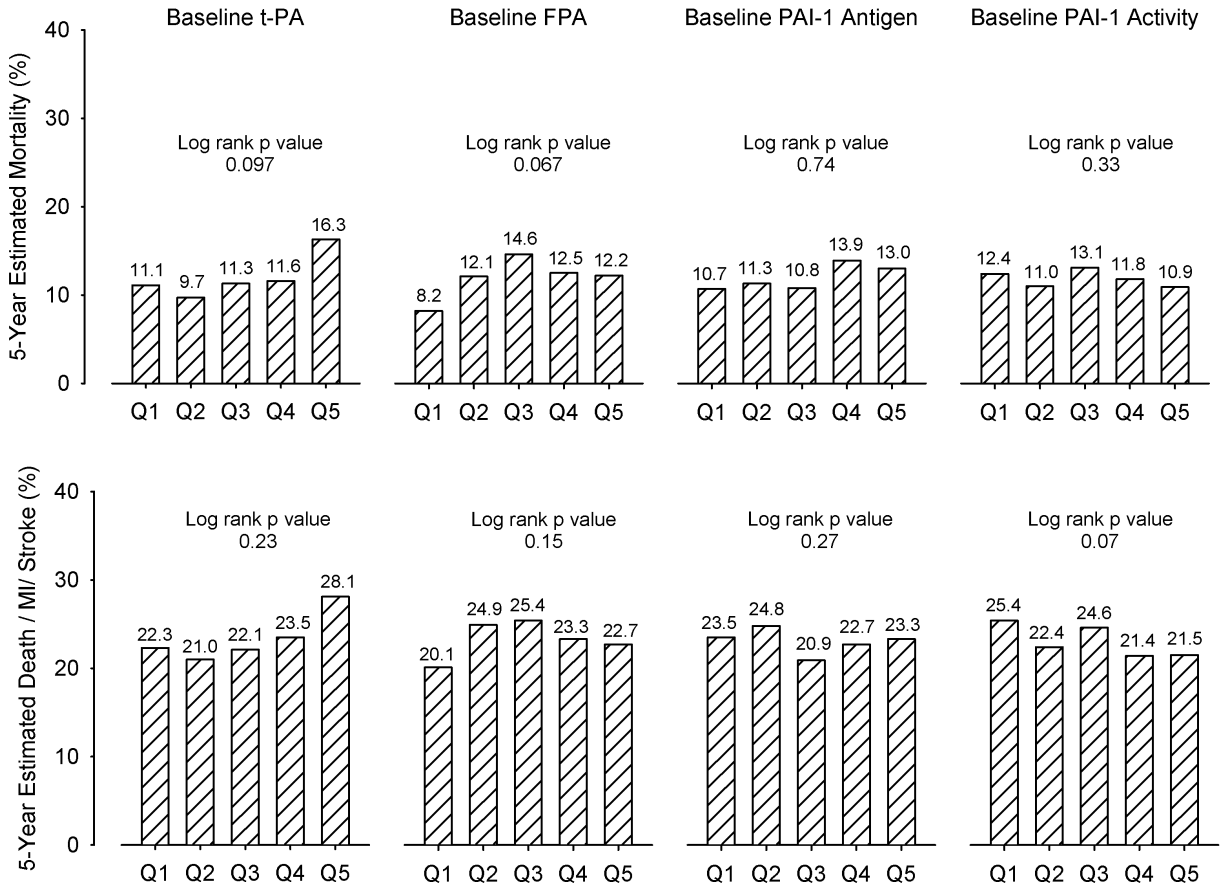

CRP
µg/ml
0
10
20
30
40
50
60
BL
1Yr
N 2283 1975
Median 2.39 1.44
IQR 4.72 2.97
D-dimer
µg/ml FEU
0
1
2
3
4
BL
1Yr
N 2279 1973
Median 0.32 0.33
IQR 0.41 0.39
Fibrinogen
mg/dl
200
400
600
800
BL
1Yr
N 2302 1980
Mean 366.0 335.4
SD 97.6 93.4
FPA
ng/ml
0
10
20
30
40
50
60
70
BL
1Yr
N 2301 1986
Median 11.0 6.9
IQR 31.8 20.3

Figure 3


Baseline t-PA
Baseline FPA
Baseline PAI-1 Antigen
Baseline PAI-1 Activity
5-Year Estimated Mortality (%)
Log rank p value
0.097
Log rank p value
0.067
Log rank p value
0.74
Log rank p value
0.33
11.1 9.7 11.3 11.6 16.3
8.2 12.1 14.6 12.5 12.2
10.7 11.3 10.8 13.9 13.0
12.4 11.0 13.1 11.8 10.9
5-Year Estimated Death / MI/ Stroke (%)
Log rank p value
0.23
Log rank p value
0.15
Log rank p value
0.27
Log rank p value
0.07
22.3 21.0 22.1 23.5 28.1
20.1 24.9 25.4 23.3 22.7
23.5 24.8 20.9 22.7 23.3
25.4 22.4 24.6 21.4 21.5
0 10 20 30 40
Q1 Q2 Q3 Q4 Q5